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Three-dimensional Migration of Neurites Is Mediated by Adhesion Site Density and Affinity*

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Three-dimensional neurite outgrowth rates within fibrin matrices that contained variable amounts of RGD peptides were shown to depend on adhesion site density and affinity. Bi-domain peptides with a factor XIIIa substrate in one domain and a RGD sequence in the other domain were covalently incorporated into fibrin gels during coagulation through the action of the transglutaminase factor XIIIa, and the RGD-dependent effect on neurite outgrowth was quantified, employing chick dorsal root ganglia cultured two- and three-dimensionally within the modified fibrin. Two separate bi-domain peptides were synthesized, one with a lower binding affinity linear RGD domain and another with a higher binding affinity cyclic RGD domain. Both peptides were cross-linked into fibrin gels at concentrations up to 8.2 mol of peptide/mol of fibrinogen, and their effect on neurite outgrowth was measured. Both two- and three-dimensional neurite outgrowth demonstrated a bi-phasic dependence on RGD concentration for both the linear and cyclic peptide, with intermediate adhesion site densities yielding maximal neurite extension and higher densities inhibiting outgrowth. The adhesion site density that yielded maximal outgrowth depended strongly on adhesion site affinity in both two and three dimensions, with lower densities of the higher affinity ligand being required (0.8–1.7 mol/mol for the linear peptide *versus* 0.2 mol/mol for the cyclic peptide yielding maximum neurite outgrowth rates in three-dimensional cultures).

Cell adhesion and migration in two and three dimensions play important roles in a host of physiological phenomena, including neurite extension (1), angiogenesis (2), and immune responses (3). The adhesion of cells within their environment is controlled primarily by the binding of cell surface receptors to short, exposed protein domains in the extracellular matrix. An important receptor family involved in adhesion and migration is the integrins, a class of heterodimeric receptors that interact with both extracellular matrix and cell-surface ligands (4, 5). Each unique dimeric receptor binds to a particular subset of ligands, which can often be mimicked with immobilized short peptides (6). One such short peptide that binds to integrins and influences subsequent cell adhesion and migration is RGD, a motif present in many proteins, including fibronectin, laminin, collagen, and fibrinogen (7). This sequence has been shown to be recognized by $\alpha_5\beta_1$, $\alpha_v\beta_3$ (8), and several other integrin heterodimer pairs.

Adhesion and migration upon a surface are distinct processes that depend on related molecular mechanisms. Cell adhesion involves the dynamic interaction between cell surface receptors and adhesion ligands on the supporting matrix (9, 10), with the strength of this attachment being related to the concentration of cell-surface receptors, the concentration of ligands available, and the specific binding affinity of the receptor ligands and pairs (11). Migration of cells upon a material is an analogous phenomenon, with the rate of cell migration depending on both ligand density and binding affinity as well as the density of corresponding cell surface receptors. As cells move within their environment, they must constantly detach at the trailing edge and reattach at the leading edge to nearby ligands (12, 13). The corresponding rate of cell migration upon a surface depends on the simultaneous detachment and attachment to nearby ligands, causing extreme ligand densities (both high and low) to inhibit one of these two processes and subsequently limit cell migration rates. The outgrowth of neurites is analogous to cell migration (14), with receptor cluster morphology (15) and receptor dynamics at the growth cone (16) controlling the process.

Cell migration and neurite extension within a three-dimensional matrix involves a more complex balance of cellular activity than migration upon a two-dimensional surface, with cell-associated proteolysis playing a key role in three-dimensional migration. When cells are present inside a fibrin matrix for example, the porosity of the fibrous network is significantly smaller than the average cell and even neurite size. Therefore, in order for cells or neurites to migrate within fibrin, it is necessary that the surrounding matrix be remodeled, and this is accomplished through the action of the protease plasmin (17–19). While adhesion signals still play an important role in three-dimensional migration, the dependence also on proteolysis complicates a one-to-one correlation between two- and three-dimensional cell migration.

The focus of this work was neurite migration within a fibrin matrix with controlled adhesive characteristics. Through the action of the transglutaminase, factor XIIIa, bi-domain peptides were incorporated within a three-dimensional fibrin matrix at precise and predetermined concentrations (20). A bi-phasic response was observed in both two- and three-dimensional neurite outgrowth, with intermediate RGD adhesion site densities leading to fastest outgrowth. Outgrowth rates depended strongly on binding affinity with a lower number of higher affinity binding sites leading to a similar response as a higher number of lower affinity ligands.

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EXPERIMENTAL PROCEDURES

Peptides and Proteins—d*LNQEQVSPL*RGD-NH_2 and d*LNQEQVSPL*RDG-NH_2 (d = dansyl; for clarity, the factor XIIIa substrate domain (21) is shown in italics) were synthesized on solid resin using an automated peptide synthesizer (9050 Pep Plus Synthesizer, Millipore, Framingham, MA) with standard 9-fluorenylmethyloxycarbonyl chemistry (22). These peptides were labeled with a fluorescent probe by including an α-dansylated leucine (Sigma, Buchs, Switzerland) at the amino terminus of the peptide. Hydrophobic scavengers and cleaved protecting groups were removed by precipitation of the peptide in cold diethyl ether and dissolution in deionized water. After lyophilization, these two peptides were redissolved in 0.03 M Tris-buffered saline (TBS,[1] pH 7.0) and analyzed using high performance liquid chromatography (Waters, Milford, CT) on a size exclusion column with TBS, pH 7.0, as the running buffer. The cyclic peptide, NH_2-L*NQEQVSP*D-[CRGDNRC] (cyclic ring shown in brackets), was purchased from Genemed (South San Francisco, CA) at greater than 85% purity.

Fibrinogen solutions were prepared by dissolving fibrinogen (Fluka, Buchs, Switzerland) in deionized water at 8 mg/ml for 4 h. Dialysis (Spectrum, 6000–8000 MWCO; Laguna Hills, CA) *versus* 400 volumes of TBS, pH 7.4, for 24 h was used to exchange salts present in the protein solution. The resulting solution was then sterilized by filtering through a 0.22-μm syringe filter. The final fibrinogen concentration was determined by measuring the absorbance of the protein solution at 280 nm (23). TBS solutions at pH 7.0 that were made with either 50 mM Ca^{2+} or without any Ca^{2+} were sterilized by filtration though a 0.22-μm filter. Fresh thrombin solutions were made by dissolving thrombin (Sigma, Buchs, Switzerland) in TBS at pH 7.0 at a concentration of 20 units/ml.

Fibrin Formation—Fibrin gels were made in flat-bottomed 24-well culture plates by mixing the four components such that final concentrations obtained were 1) 3.6 mg/ml fibrinogen, 2) 2.5 mM Ca^{2+}, 3) 2 NIH units/ml of thrombin, and 4) various amounts of added peptide.

DRG Dissection—DRGs were dissected from 8-day-old White Leghorn chicken embryos (24). Ganglia were temporarily stored in modified neurobasal medium, consisting of insulin (5 μg/ml), transferrin (100 μg/ml), progesterone (6.3 ng/ml), putrascine (16.11 μg/ml), selenite (5.2 ng/ml), fibronectin (5 μg/ml), L-glutamine (0.5 mM), L-glutamate (25 μM), murine mouse nerve growth factor (20 ng/ml), 0.2% bovine serum albumin, and 1% antibiotic-antimycotic added to neurobasal medium (Life Technologies, Inc.).

Competitive Inhibition of Three-dimensional Neurite Outgrowth—DRGs were cultured within 400 μl of unmodified fibrin gels, with the gels being polymerized in the presence of a DRG such that one ganglion was embedded three-dimensionally within the fibrin gel during polymerization. After polymerization, 1 ml of modified medium was added to the matrices. In three series of experiments, the medium added after fibrin gel polymerization was further supplemented with either of the RGD peptides or the control RDG peptide added as soluble competitive inhibitors. In the first experiment, the linear RGD peptide was added to the medium at concentrations of 0, 0.21, 0.42, and 1.05 mM. The peptide-supplemented medium was changed at 3, 6, and 9 h after seeding, and the migration of neurites at 24 h was quantified and normalized to that observed when 0 mg/ml soluble peptide was added. A second series of experiments was performed in which the cyclic RGD peptide was added as a competitive soluble inhibitor at the concentrations of 0, 0.08, 0.16, or 0.40 mM. Finally, a third series of experiments was performed with the linear RDG peptide, where the peptide was added to the medium at concentrations of 0, 0.21, 0.42, and 1.05 mM. The washing and analysis procedures were identical to those used with the linear RGD peptide, with the peptide-supplemented medium changed at 3, 6, and 9 h. Neurite outgrowth was measured as described below.

Two-dimensional Neurite Outgrowth—DRGs were cultured upon fibrin gels without added exogenous peptides (all values were normalized to this level of neurite extension within native fibrin) or with cross-linked d*LNQEQVSPL*RGD-NH_2, d*LNQEQVSPL*RDG-NH_2 or L*NQEQVSP*D[CRGDNRC]. 300-μl gels were synthesized with various amounts of peptide and washed at 3, 6, 9, and 24 h after gelation with TBS to remove noncross-linked peptide. The peptides employed were cross-linked at different densities. The linear RGD and RDG were incorporated separately in a series of final concentrations that corresponded to 0.8, 1.7, 4.4, and 8.2 mol of peptide/mol of fibrinogen, obtained by polymerization of fibrinogen in the presence of 55, 110, 220, 275, and 330 μM peptide (20). The cyclic RGD was incorporated at 0.2, 0.4, 4.4, and 8.2 mol of peptide/mol of fibrinogen, obtained by polymerization of fibrinogen in the presence of 13.75, 27.5, 275, and 330 μM peptide. DRGs were placed on the surface of the fibrin gel and allowed to adhere for 2 h before medium was added, and then the ganglia were cultured on the surface of the matrix for 24 h. The neuronal medium used was further modified by the addition of 1 μg/ml aprotinin to prevent the ganglia from detaching from the surface due to protease activity (17). Neurite extension was measured at 24 h after seeding as described below.

Three-dimensional Neurite Outgrowth—DRGs were cultured within 400-μl fibrin gels without added exogenous peptides (all values were normalized to this level of neurite extension within native fibrin) or with cross-linked d*LNQEQVSPL*RGD-NH_2, d*LNQEQVSPL*RDG-NH_2, or L*NQEQVSP*D[CRGDNRC]. These gels were polymerized in the presence of a DRG such that one ganglion was embedded three-dimensionally within the fibrin gel during polymerization. All peptides were incorporated separately in a series of final concentrations that corresponded to 0.8, 1.7, 4.4, and 8.2 mol of peptide/mol of fibrinogen, obtained by polymerization of fibrinogen in the presence of 55, 110, 220, 275, and 330 μM peptide (20). The cyclic peptide was cross-linked into the gel at two additional concentrations of 0.2 and 0.4 mol of peptide/mol of fibrinogen, obtained from the addition of 13.75 and 27.5 μM peptide during polymerization. The fibrin gels with embedded ganglia were cultured in 1 ml of modified neurobasal medium, and the medium was changed at 3, 6, 9, and 24 h after seeding to wash out noncross-linked peptide. Neurite outgrowth was measured at 24 and 48 h after seeding as described below.

Imaging—Images of the entire DRG and the outgrowing neurites were collected using phase contrast microscopy with a $\times$ 4.0 objective (Zeiss, Zurich, Switzerland). At each time point, the average neurite length extending from each DRG was measured by digital image processing as the area-averaged distance between the edge of the corona of extending neurites and the DRG body, measured as described in detail elsewhere (17).

Confocal scanning laser microscopy of DRGs was performed at 24 h with a 10×/0.30 Plan Neofluor objective (Zeiss) using a MRC 600 confocal system (Bio-Rad, Glattbrugg, Switzerland). 200-μl fibrin gels containing various amounts of linear or cyclic RGD peptide or linear RDG peptide were polymerized with a DRG within the gel in the center of a 35-mm Petri dish. The DRGs were cultured in 2 ml of neuronal medium, which was replaced once at 6 h to remove free peptide. Samples were stained at 24 h, prior to imaging, by adding 2 μl of a 5 mg/ml stock solution of fluorescein diacetate in acetone to 1 ml of TBS. Approximately 50 images were taken at 10-μm intervals, and a composite image was assembled using Imaris (Bitplane, Zurich, Switzerland) image processing software on an Indigo2 extreme Silicon Graphics Workstation (Silicon Graphics, Mountain View, CA).

Statistics—The outgrowth for each individual ganglion was normalized by the mean neurite outgrowth observed for ganglia simultaneously cultured in or on unmodified fibrin. Experiments were completed in triplicate and these normalized values were then averaged to express the change in outgrowth in relation to outgrowth observed to unmodified fibrin. Statistical analyses of these data were performed using Statview 4.5 (Abacus, Berkeley, CA). Comparative analyses were completed using analysis of variance with Fisher's protective t test at a 95% confidence level. Mean values and standard errors of the mean are shown.

Linear curve fits were calculated for the data on inhibition with soluble peptides using Cricket Graph III (Computer Associates), which employs a linear least squares regression method. The analysis was limited to the linear domain of the inhibition curves and was used to determine the relative binding affinities of the two RGD peptides employed.

RESULTS

Competitive Inhibition of Three-dimensional Neurite Outgrowth—As a measure of the relative binding affinity of the two RGD peptides employed, the effect of each peptide as a soluble competitive inhibitor of three-dimensional neurite outgrowth from day 8 chick DRGs within fibrin was studied. When the linear peptide, d*LNQEQVSPL*RGD, was added to the culture medium, after fibrin polymerization, as a competitive inhibitor in a concentration series, neurite outgrowth decreased in a dose-dependent manner (Fig. 1). At the highest concentration tested, 1.05 mM, the peptide decreased neurite outgrowth by 25 $\pm$ 7% at 24 h relative to the outgrowth within unmodified

[1] The abbreviations used are: TBS, Tris-buffered saline; DRG, dorsal root ganglia.

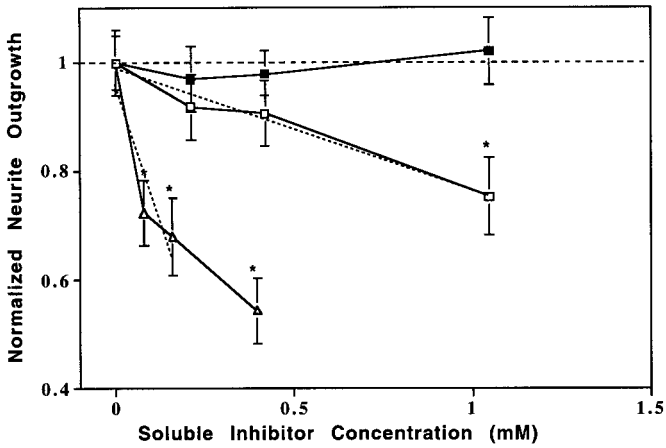

FIG. 1. **The effect of soluble cyclic (△) or linear (□) RGD peptides and a linear (■) RDG control peptide on neurite outgrowth within three-dimensional fibrin matrices.** Peptide was added in the culture medium as a soluble competitive inhibitor for endogenous RGD in the fibrin matrix. Neurite outgrowth from day 8 DRGs was measured and normalized to outgrowth in unmodified fibrin at 24-h culture time (nonnormalized value for unmodified fibrin is 0.207 ± 0.010 mm). Experiments were performed in triplicate with six DRGs per replicate, and mean values and S.E. are shown; * denotes $p < 0.05$ compared with unmodified fibrin. At each concentration point, the cyclic peptide statistically inhibited neurite outgrowth to a greater extent than did the linear peptide, demonstrating that the cyclic RGD is a higher affinity ligand than the linear RGD. The incorporation of the inactive control, RDG, did not affect neurite outgrowth at any of the concentrations tested. Curve fits were calculated for the two RGD peptides and are shown in *dashed lines* over the concentrations points used in this calculation. The ratio of the binding affinities for the cyclic RGD to the linear RGD (the ratio of the slope of the two linear curve fits) is 9.0.

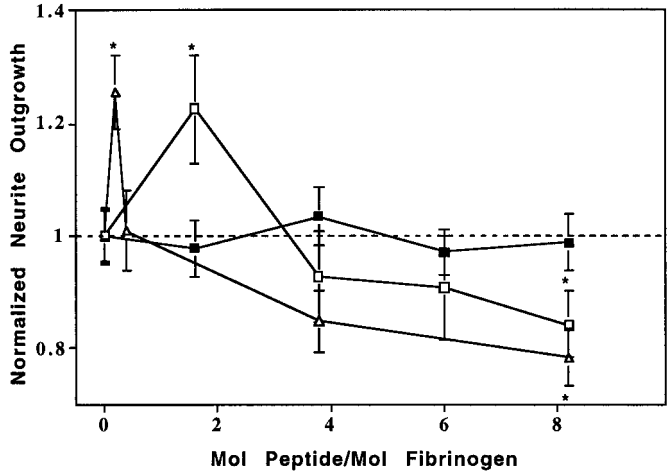

FIG. 2. **The effect of incorporated cyclic (△) or linear (□) RGD peptides and a linear (■) RDG peptide on neurite outgrowth upon two-dimensional fibrin matrices.** Neurite outgrowth from day 8 DRGs was measured and normalized to outgrowth in unmodified fibrin at 24 h culture time (nonnormalized value for unmodified fibrin is 0.238 ± 0.010 mm). Experiments were performed in triplicate with six DRGs per replicate, and mean values and S.E. are shown; * denotes $p < 0.05$ compared with unmodified fibrin. The correlation between two-dimensional neurite outgrowth and RGD density showed a bi-phasic effect, with moderate densities of exogenous peptide eliciting the greatest neurite outgrowth. The incorporation of the inactive control peptide, RDG, did not affect two-dimensional neurite outgrowth at any of the concentrations tested.

fibrin in the absence of soluble peptide inhibitor ($p < 0.05$). The cyclic peptide L*NQEQVSP*D[CRGDNRC] decreased neurite outgrowth to a greater extent compared with the linear peptide, *e.g.* by 46 ± 6% at 24 h when 0.40 mM peptide was added to the medium. Furthermore, at every concentration point, the cyclic RGD peptide inhibited the growth of neurites to a greater extent than did the linear RGD peptide ($p < 0.05$). Linear curve fits were calculated for the two active RGD peptides, and it was found that the slope for the curve fit for the cyclic RGD peptide was 9.0 times steeper than that for the curve fit for the linear RGD peptide. The control sequence, RDG, proved to have no effect on three-dimensional neurite outgrowth over the entire concentration range tested ($p > 0.52$).

Neurite Extension in Two Dimensions with Cross-linked RGD Sites—Fibrin gels were modified by cross-linking various concentrations of either the linear RGD peptide, the cyclic RGD peptide or the linear control RDG peptide, and a bi-phasic RGD-dependent effect on two-dimensional neurite outgrowth was observed (Fig. 2). When linear RGD was incorporated over the range of 0–8.2 mol of peptide/mol of fibrinogen, the cross-linking of 1.7 mol of linear RGD/mol of fibrinogen proved to provide the greatest enhancement of neurite outgrowth, increasing the level of neurite outgrowth by 23 ± 9% at 24 h ($p < 0.05$). Neurite outgrowth decreased for each of the higher peptide densities such that when 8.2 mol of linear RGD/mol of fibrinogen was incorporated the level of neurite outgrowth decreased by 16 ± 6% of the level seen in unmodified fibrin matrices ($p < 0.05$). The incorporation of the cyclic RGD peptide over the same concentration range demonstrated a similar bi-phasic effect on neurite outgrowth, with the maximal enhancement occurring at a lower cross-linked peptide density. When 0.2 mol of cyclic RGD/mol of fibrinogen was incorporated, the level of enhancement proved to be 26 ± 7% compared with unmodified fibrin. Once again, at each of the higher peptide densities, the level of neurite outgrowth decreased such that at 8.2 mol of cyclic RGD/mol of fibrinogen, the level of neurite outgrowth was reduced by 22 ± 5% of the level observed in unmodified fibrin. The control sequence, RDG, proved to not have an effect on neurite outgrowth over the entire concentration range tested, with the level of outgrowth similar to that in unmodified fibrin ($p > 0.74$).

Neurite Extension in Three Dimensions with Cross-linked RGD Sites—Fibrin gels were modified by cross-linking various concentrations of linear or cyclic RGD or linear RDG peptide into the gel, and the resulting effect on three-dimensional neurite outgrowth was measured at 24 and 48 h. A bi-phasic response was observed, with the cross-linking of 1.7 mol of linear RGD peptide/mol of fibrinogen enhancing the level of neurite outgrowth by 23 ± 7% at 24 and 14 ± 5% at 48 h ($p < 0.05$) (Fig. 3). The incorporation of 4.4 or 8.2 mol of linear RGD peptide/mole fibrinogen decreased the level of neurite outgrowth, such that it was lower than the outgrowth observed within both unmodified fibrin as well as the fibrin modified with lower concentrations of cross-linked peptide ($p < 0.05$). At the highest incorporated adhesion density of 8.2 mol/mol, the level of neurite outgrowth was reduced by 21 ± 5% at 24 and 13 ± 4% at 48 h relative to outgrowth within unmodified fibrin.

The incorporation of the higher affinity-binding cyclic peptide, L*NQEQVSP*D[CRGDNRC], into fibrin gels had a similar bi-phasic effect on the outgrowth of neurites, with an overall effect that was shifted to lower peptide densities relative to the effect for the lower affinity linear RGD peptide. Incorporation of 0.2 mol/mol of cyclic peptide enhanced the outgrowth of neurites by 17 ± 7% at 24 and 14 ± 3% at 48 h compared with outgrowth within unmodified fibrin ($p < 0.05$). Each of the higher cross-linking concentrations tested resulted in a decrease in neurite outgrowth, such that at 0.8 mol/mol, outgrowth was reduced by 25 ± 7% at 24 and 16 ± 4% at 48 h, and at 8.2 mol/mol, neurite outgrowth was reduced by 51 ± 5% at 24 and 25 ± 5% at 48 h relative to outgrowth within unmodified fibrin ($p < 0.05$). Incorporation of the RDG peptide had no

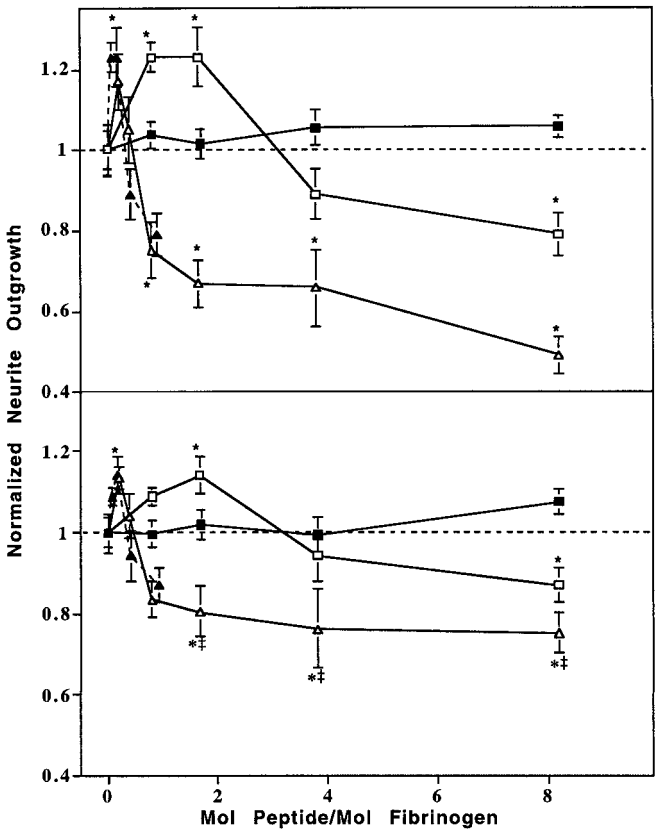

FIG. 3. **The effect of incorporated cyclic (△) or linear (□) RGD peptides and a linear (■) RDG peptide at various concentrations on neurite outgrowth within three-dimensional fibrin matrices.** Neurite outgrowth from day 8 DRGs was measured and normalized to outgrowth in unmodified fibrin at 24 (*A*) and 48 h (*B*) culture time (nonnormalized value for unmodified fibrin is 0.207 ± 0.010 mm at 24 h and 0.746 ± 0.027 mm at 48 h). Experiments were performed in triplicate with six DRGs per replicate, and mean values and S.E. are shown; * denotes $p < 0.05$ compared with unmodified fibrin, and ‡ denotes points that are statistically identical. The correlation between neurite outgrowth and cross-linked linear RGD peptide showed a similar bi-phasic effect as seen in two-dimensional RGD-dependent cellular migration. The incorporated cyclic RGD peptide showed a similar effect, with the curve shifted consistently to lower concentrations due to its higher binding affinity. The relative binding affinity ratio calculated from the curve fits of the competitive soluble inhibition experiment (Fig. 1) was used to normalize the linear RGD data at both 24 and 48 h (▲). When the linear RGD data are normalized in this manner, both the 24- and 48-h neurite outgrowth are very similar to that observed with the cyclic RGD peptide. The incorporation of the inactive peptide, RDG, did not affect neurite outgrowth at any of the concentrations.

statistically significant effect on neurite outgrowth at either 24 or 48 h at any of the concentrations tested ($p > 0.36$).

The relative binding affinities of the cyclic and the linear peptides were estimated from the results on competitive inhibition in three-dimensional outgrowth experiments. The cyclic peptide was estimated to bind 9.0 times more strongly. This ratio was used to compare the results in outgrowth with immobilized linear and cyclic peptides in terms of affinity, rather than molar amounts of peptide, by dividing the abscissa for the linear peptide by 9.0. The adjusted results are shown in Fig. 3. The curves, when adjusted according to relative affinity are highly coincident.

The difference in outgrowth induced from the ganglia in the various samples was pronounced enough to be clearly observed. As a demonstration, a representative sample from materials that induced the greatest and the smallest neurite outgrowth are shown in Fig. 4. Samples are shown for the linear peptide where maximal outgrowth (*C*) and minimal outgrowth (*E*) were achieved. An identical set are present for the cyclic peptide (*D* and *F*, respectively) in addition to representative samples for the two controls, unmodified fibrin (*A*) and RDG modified fibrin (*B*).

DISCUSSION

The migration of cells within a three-dimensional matrix is an important component of many cellular processes both *in vivo* and *in vitro*. However, the lack of three-dimensional model materials in which the biochemical characteristics can be readily and reproducibly controlled prevents complete understanding of the relevant factors involved. Through the factor XIIIa-mediated modification of fibrin matrices, we have developed a method by which the bioactive character of a degradable matrix can be reproducibly manipulated (20). Using these matrices, the influence that exogenous RGD sequences have on the outgrowth of neurites within a three-dimensional environment was studied, with the effect on neurite outgrowth shown to exhibit a bi-phasic dependence on RGD concentration and binding affinity.

The relative binding affinities of the peptides employed were characterized by competitive inhibition experiments in three-dimensional culture. RGD is found endogenously in fibrin, at an active concentration of 2 mol/mol of fibrinogen (25). The addition of either of the RGD peptides as a soluble inhibitor blocked the activity of these endogenous sequences, resulting in a decrease in neurite outgrowth within the fibrin matrix. A high concentration of the linear RGD peptide (1.05 mM) was required to decrease the outgrowth of neurites. The addition of the cyclic RGD peptide as a soluble competitive inhibitor proved to be much more potent in slowing neurite outgrowth, such that at each tested concentration, the cyclic peptide inhibited neurite outgrowth to a greater extent than did the linear peptide. When the data were correlated to a linear curve fit, it was shown that the curve fit for the cyclic peptide was 9.0 times steeper then that for the linear RGD, indicating that the binding affinity in this system for the cyclic peptide is 9.0 times greater than that for the linear RGD peptide. This greater binding affinity for cyclic RGD peptides over linear RGD peptides has been demonstrated previously (26), with the enhanced binding affinity due to the forced secondary structure occurring from the ring (27). It has been shown that cyclic RGD peptides adopt a conformation similar to the turn between strands in a β sheet, forcing cyclic peptides to mimic a common structural motif for native RGD sites (28).

Two-dimensional neurite outgrowth demonstrated a bi-phasic dependence on RGD site density, consistent with the outgrowth of neurites being a form of cell migration. Much experimental and modeling work has been completed showing that the effect of adhesion site surface concentration on the motility of cells, such as smooth muscle cells, displays a bi-phasic response (12, 29). The extent of the bi-phasic response in two dimensions depends on the adhesion site and receptor surface concentrations and the degree to which cell surface receptors are polarized toward the leading edge (11). Evidence suggests that the migration of the neuronal growth cone, and therefore the overall neurite outgrowth rate, represents a similar phenomenon (16). By incorporating RGD into a fibrin matrix, it has been demonstrated that the neurite outgrowth from chick DRGs displays such a bi-phasic dependence on RGD density, with a moderate peptide density eliciting the highest outgrowth rate. These results are thus consistent with the migration of neuronal growth cones, and therefore neurite outgrowth, being directly analogous to the migration of individual cells.

RGD peptides cross-linked into fibrin gels demonstrated a bi-phasic adhesive-like character in three-dimensions. The

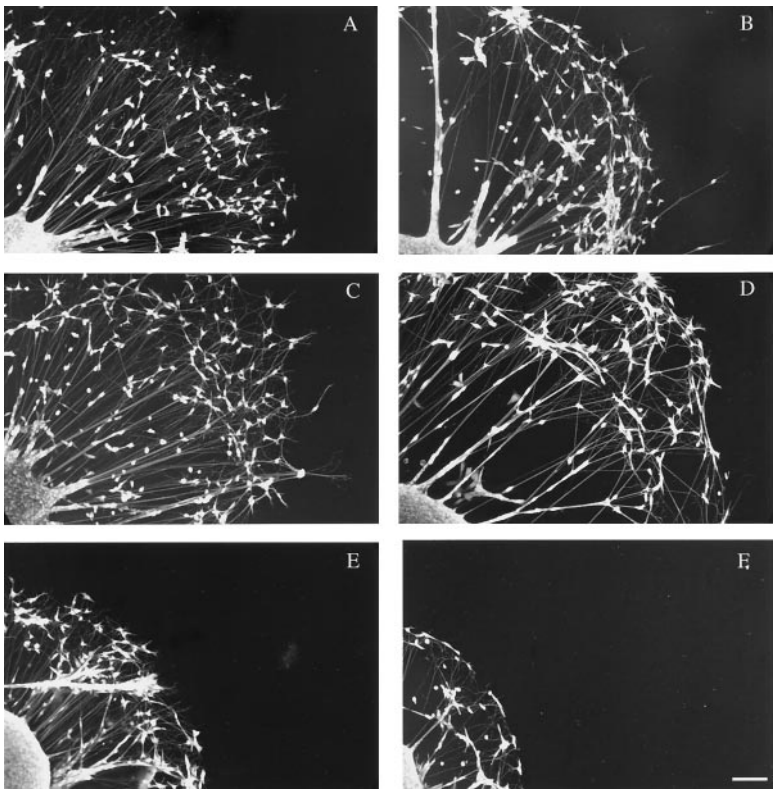

FIG. 4. **Images of DRGs cultured within unmodified fibrin (*A*), fibrin with 8 mol of RDG/mol of fibrinogen (*B*), fibrin with 1.7 mol of linear RGD/mol of fibrinogen (*C*), fibrin with 0.2 mol of cyclic RGD/mol of fibrinogen (*D*), fibrin with 8 mol of linear RGD/mol of fibrinogen (*E*), and fibrin with 8 mol of cyclic RGD/mol of fibrinogen (*F*).** Confocal scanning laser microscopy of DRGs was performed using 10x magnification with approximately 50 images taken at 10-μm intervals shown as an extended focus projection. The *scale bar* represents 100 μm. The ganglia grown within unmodified fibrin show a similar level of outgrowth to that seen within RDG-modified fibrin gels. This level is less than that observed in ganglia grown within fibrin with low levels of incorporated RGD peptides (*C* and *D*) but greater than that observed in ganglia grown within fibrin with high levels of incorporated RGD peptides (*E* and *F*).

striking similarity between the results in three-dimensional and two-dimensional outgrowth experiments suggest that the rate-controlling phenomena are similar between the two cases. This is very interesting, because in two-dimensional cultures proteolysis plays a minor role, whereas in the three-dimensional cultures proteolysis is required for neurite outgrowth (17). The similarity in results suggests that proteolysis of the fibrin matrix is not the rate-controlling phenomenon, but rather that adhesion and migration biomechanics control the overall outgrowth rate. The results also suggest, although indirectly, that the density of exogenous RGD sites did not influence neurite outgrowth rates primarily by modulating local protease activity. If they had done so, then the site density-dependent bi-phasic response that was observed in three-dimensional outgrowth (where proteolysis is requisite) would not have been observed in two-dimensional outgrowth (where proteolysis is not requisite) (17).

A strong dependence of both two-dimensional and three-dimensional neurite outgrowth upon adhesion site binding affinity was observed. The phenomena observed for the two RGD peptides employed were similar in both form and extent, but with the response curve being markedly shifted to lower concentrations for the higher affinity cyclic RGD peptide such that a significantly lower incorporated density of cyclic RGD was required to induce maximal neurite outgrowth. To better quantify this phenomenon, the three-dimensional outgrowth was presented for the two RGD peptides in terms of comparable adhesive density rather than peptide density. This was accomplished by first calculating the relative binding affinities for the two RGD peptides from the competitive inhibition study. This ratio was calculated to be 9.0 and was then used to correlate the effects of the two RGD sequences by dividing the incorporated density for the linear peptide by this relative binding affinity ratio. When this was done, the RGD-dependent neurite outgrowth for the adjusted linear RGD peptide (Fig. 3, ▲) was strikingly similar to that for the cyclic RGD peptide (Fig. 3, △). Therefore, it is very likely that the lower density of the higher affinity cyclic peptide, which induced maximal neurite outgrowth represents a total adhesive character similar to the higher density of the lower affinity linear peptide, which induced maximal neurite outgrowth as well. This is further supported in that the levels of enhancement of neurite outgrowth for these two formulations are of similar intensities. Within the concentration series tested, both RGD peptides demonstrated a similar adhesive-like character in three-dimensional migration, and this character corresponds directly to effects seen in the two-dimensional studies.

It has been recently demonstrated that integrin number in the growth cone is regulated by receptor occupancy. Neurons were purified from embryonic chick DRGs and plated on surfaces coated with either a low or high concentration of the extracellular matrix protein laminin or the extracellular matrix protein fibronectin. When the amount of a laminin-specific integrin receptor, $\alpha_6\beta_1$, was analyzed, it was shown to have increased in the presence of low concentrations of laminin and decreased in the presence of high concentrations of laminin relative to similar neurons plated on fibronectin coated surfaces (30). This compensatory mechanism is hypothesized to enable the neurite to migrate over a very wide range of adhesion site densities. This compensatory mechanism may be less at play in the fibrin model employed, as a clear response of outgrowth rate to the adhesive characteristics of the matrix was observed. It also may be possible that this compensatory effect is even less at play in three-dimensional outgrowth, since high immobilized concentrations of the high affinity cyclic RGD ligand inhibited neurite outgrowth much more extensively in three-dimensional culture than in two-dimensional culture, approximately 2-fold more.

The addition of exogenous adhesion sites within a fibrin matrix is sufficient for enhancing two- and three-dimensional neurite outgrowth. The overall process of neurite outgrowth involves the simultaneous coordination of many individual processes, including binding to both adhesion and other bioactive sites, polymerization of actin, formation of new membrane, and

intracellular migration of proteins within the axon (31–33). While each of these components are necessary for neurite migration, it is possible that modulating a single factor might not be sufficient to increase the overall rate of neurite migration. By cross-linking various concentrations of exogenous RGD peptides within a fibrin matrix formed from physiological fibrinogen concentrations, materials have been developed whereby the likely change is in the adhesive character of the material. These materials were able to modestly increase the level of neurite outgrowth, demonstrating that adhesion of the migrating neurite growth cone is a significant and rate controlling factor, even if it constitutes only one part of a complicated process.

The use of biomaterials for tissue regeneration, such as the promotion of nerve healing, is a promising field of research. By developing materials with specific adhesive characteristics, it is possible to control and enhance the level of cell migration within the material. This may lead to more effective forms of therapy in many areas of tissue engineering, such as promoting better integration at the tissue-biomaterial interface, promoting cell infiltration, and controlling morphogenesis in tissue regeneration.

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